SF 298 MASTER COPY KEEP THIS COPY FOR REPRODUCTION PURPOSES

REPORT DOCUMENTATION PAGE

Form Approved OMB NO. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 12, 1998	Final Progress report, April 95-April 98

4. TITLE AND SUBTITLE
Differential Gene Expression in Mammalian Liver during Hibernation

5. FUNDING NUMBERS
DAAH04-95-1-0186

6. AUTHOR(S)
Sandra L. Martin, PhD, PI

7. PERFORMING ORGANIZATION NAMES(S) AND ADDRESS(ES)
University of Colorado Health Sciences Center
4200 E. Ninth Ave.
Denver, CO 80262

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSORING / MONITORING AGENCY REPORT NUMBER
ARO 33873.3-LS

11. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

12 b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Hibernation is an adaptive strategy employed by some mammals to escape the cold. During hibernation, heart, respiratory and metabolic rates plummet, and core body temperatures can approach freezing. Hibernators spontaneously arouse using only endogenously-generated heat. Our long-range goal is to understand the molecular underpinnings of this phenotype in the context of the entire organism. Our efforts focussed upon liver because of its key role in metabolic and other aspects of physiological homeostasis. Specifically, our goals were to: 1) Determine whether there is a reversible inhibition of the oxidative phosphorylation apparatus during torpor (and, if so, whether this inhibition can explain the metabolic changes that accompany entrance and arousal from torpor), and 2) Isolate and analyze hibernation-induced genes. State 3 respiration is reversibly suppressed during torpor. However, this suppression was not found to be causally linked to entrance and arousal. Our other studies identified serum amyloid P as differentially expressed during hibernation. Both it and alpha-2-macroglobulin (also demonstrated to be upregulated during hibernation) apparently play a role in lowering the efficiency of blood clotting. Reduced clotting during hibernation has been recognized for many years and is thought to be critical for survival at the low heart rates that accompany hibernation.

14. SUBJECT TERMS	15. NUMBER IF PAGES
hibernation, ground squirrels, liver, gene expression, state 3 and state 4 respiration, mitochondria	6
	16. PRICE CODE

17. SECURITY CLASSIFICATION OR REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

REPORT DOCUMENTATION PAGE (SF298)
(Continuation Sheet)

FINAL PROGRESS REPORT

DIFFERENTIAL GENE EXPRESSION IN MAMMALIAN LIVER DURING HIBERNATION

Statement of the problem studied.

The long range goal of our studies is to understand the molecular basis for the extraordinary reductions in heart rate, core body temperature and metabolism that are seen in mammals during hibernation. If we can define the biochemical means for control and survival of this remarkable state in natural hibernators, it may be possible to mimic aspects of hibernation in humans. Liver is a key organ in maintenance of homeostasis in all mammals; this role is likely to be amplified during hibernation. Thus, our work was focussed upon liver. Specifically, the aims of the recently concluded project were to (1) examine changes in mitochondrial function during hibernation and (2) isolate and identify novel gene products that are differentially expressed during hibernation.

Summary of the most important results.

1. ***Role of increased mitochondrial gene products during hibernation.*** This project was stimulated by results obtained during an earlier funding period. Subtractive hybridization of hibernating liver cDNA enriched a number of genes which function in oxidative phosphorylation, suggesting that these gene products are upregulated for hibernation. This is a somewhat paradoxical result, given the low metabolic rates that characterize the torpid phases that dominate the hibernating state. The literature concerning the activity of mitochondrial oxidative phosphorylation during hibernation was confusing and contained conflicting reports of decreased, increased or unchanged activity (reviewed in Gehnrich and Aprille, 1988). Attempting to resolve the controversy, we began to examine the respiratory properties of isolated mitochondria from various phases of the hibernation cycle, using animals whose state was known precisely by body temperature telemetry. For the first set of experiments, oxygen consumption by isolated mitochondria from summer active, torpid and interbout arousal animals was measured. Significant differences in the rate of fully-coupled respiration (state 3) were observed, with torpid hibernators being repressed relative to summer active or interbout aroused hibernators. In contrast, there were no significant differences in the rates of state 4 respiration among animals representing these three states, indicating that proton leak is not regulated as a function of hibernation. In every case, mitochondria from torpid animals were isolated and analyzed in parallel with mitochondria from interbout aroused animals, in an effort to eliminate potential artifacts generated by sample handling. Whatever the biochemical mechanism, the observed inhibition of oxygen consumption is stable enough to survive isolation and assay of mitochondria *in vitro* over a period of several hours, yet it is fully reversed during similar periods of time *in vivo* during natural arousal from hibernation.

These results raised the exciting possibility that inhibition of state 3 respiration could be a key regulator of the entrance into torpor. The question of how hibernators effect the reduction of body temperature that is required for entrance into torpor is hotly debated, with hypotheses ranging from cooling being the result of passive Q_{10} effects (Geiser, 1988), to the result of an active mechanism to suppress metabolism (Heldmaier and Ruf, 1992). Given our demonstration that there is an active but reversible suppression of state 3 respiration during torpor, it seemed plausible that this could be an important mechanism for generation of the metabolic suppression that drives entrance into torpor. If inhibition of state 3 respiration causally related to entrance into torpor, then that inhibition should precede the reduction of body temperature. In addition, it is likely that this mechanism would need to be reversed during the arousal process, before body temperature has returned to 37°C. To test this hypothesis, we isolated mitochondria from animals at 20°C as they were entering or emerging from a torpor bout. Our data clearly refute this hypothesis: namely, values of state 3 respiration obtained from liver mitochondria isolated from entrance animals did not differ significantly different from values obtained from interbout aroused animals, but were different from torpid hibernators. Thus, we conclude that suppression of state 3 respiration, although it is present during torpor, is not a key mechanism used by hibernators to suppress metabolic rate during entrance into torpor. We have submitted this work for publication (Martin et al., 1998).

2. *Isolation and analysis of hibernation-induced genes in the liver.* Our original approach to this problem involved subtractive hybridizations to selectively deplete a hibernating liver cDNA library of sequences that were shared with a cDNA library from an active animal. This approach proved to be unfruitful because it led only to a collection of false-positives (the oxidative phosphorylation genes discussed above), so we next tried a differential display-polymerase chain reaction (dd-PCR) strategy. Although we did identify two differentially expressed genes, this approach was frustratingly inefficient. The source of RNA used for the dd-PCR was liver from two summer active golden-mantled ground squirrels and two animals in the process of re-entering torpor (following an interbout arousal) during the hibernation season. With this method, we isolated, cloned and partially sequenced two gene products, one of which was confirmed by Northern blot analysis to upregulated at the mRNA level during hibernation, and the other downregulated. The upregulated mRNA is the ground squirrel homologue of serum amyloid P component. Like the other protein that we previously found to be upregulated in hibernation (alpha-2-macroglobulin), serum amyloid P may function in the regulation of blood coagulation and is an acute phase reactant in some species. The downregulated mRNA is a member of a multigene family that includes chlordecone reductase, 20-alpha-hydroxysteroid dehydrogenase, and dihydrodiol dehydrogenase. The modified expression pattern of this gene is most likely due to the animals' lack of exposure to plant alkalyoids during the long winter fast, and, thus is secondarily related to hibernation. These experiences using dd-PCR were disappointing and led us to employ a different subtraction-based technique to isolate additional gene products whose expression is altered during hibernation.

We also quantified the steady-state levels of serum amyloid P, actin, albumin and alpha-2-macroglobulin mRNAs using quantitative slot blot hybridization analysis. This was done to address

our hypothesis that gene expression is generally suppressed during low body temperature, requiring the animals to periodically arouse to synthesize more mRNA and proteins in order to sustain/survive the torpid phase of hibernation. The results obtained from these studies are most consistent with the interpretation that there is a burst of mRNA synthesis during interbout arousal, followed by maintenance of those steady-state levels throughout the hypothermic phase. A significant loss of mRNA appears to occur during the arousal process, before body temperature recovers and mRNA biosynthesis resumes. A preliminary account of this work was presented at the International Hibernation Meeting (July 1996, Tasmania) and published in the Symposium Proceedings (Martin et al., 1996). A manuscript with a more complete presentation of this data is presently in preparation.

Most recently, we have begun to use high-resolution, two-dimensional protein gels to assess the complexity of differential gene expression in the liver and initiate new screens to isolate differentially expressed mRNAs using a new commercial product from Clontech, PCR-Select. The protein gels resolved nearly 1000 polypeptides, which constitutes approximately one-tenth of the known liver gene expression. The majority appeared to remain constant between the active and hibernating states, about 10% were decreased during hibernation, and about 3% were increased during hibernation. Extrapolating these ratios to the ~10,000 gene products that are expressed in liver suggests that approximately 300 proteins will be seasonally regulated for hibernation. We are particularly interested in the polypeptides that are induced during hibernation because these are the proteins that are most likely to be crucial for maintenance or survival of hibernation. In order to identify the genes that are upregulated, we have recently begun using PCR-Select, which allows cloning of genes based upon differential expression of their mRNAs. Once isolated, these clones can be characterized in detail to quantify the level of differential expression and identify the gene corresponding gene through nucleotide sequence determination. In just a short time, this method has proven much more efficient and fruitful than our previous approaches, including dd-PCR and cDNA subtraction. To date, 48 clones obtained by PCR-Select have been screened by hybridization analysis, and 12 of these were confirmed to be upregulated (this number includes redundancies in the form of multiple isolates of the same gene fragment as well as different fragments of the same gene). Three of the distinct, upregulated mRNAs are homologous to known human genes, including thyroxine-binding globulin, hemopexin, and NGAL, a lipocalin. This analysis has just begun and will be continued in the current funding period.

Publications during this funding period.

Srere, H.K., Belke, D., Wang, L.C.H., and Martin, S.L. (1995) α_2-Macroglobulin Gene Expression is Independent of the Acute Phase Response during Hibernation in Ground Squirrels. *Amer. J. Physiol.* **268**, R1507 - R1512.

Martin, S.L. Maniero, G.D. and Carey, C. (1996) Patterns of Gene Expression in the Liver during Hibernation in Ground Squirrels. in *Adaptations to the Cold.* Geiser, F., Hulbert,

A.J. and Nicol, S.C., eds. University of New England Press, Armidale, Australia, pp. 327-332.

Carey, H.V. and Martin, S.L. (1996) Preservation of Intestinal Gene Expression during Hibernation, *Amer. J. Physiol.* 271: G805-G813.

Carey, H.V. and Martin, S.L. (1996) Hibernation and the Stress Response. in *Adaptations to the Cold.* F., Hulbert, A.J. and Nicol, S.C., eds. University of New England Press, Armidale, Australia, pp. 319-325.

Scientific personnel supported by this project during this funding period.

Sandra L. Martin, Ph.D., PI
Julie Saxton, B.S., PRA
Greg Maniero, B.S., PRA
Stephanie Trelogan, B.S., M.S. (Master's degree awarded Dec. 1995)
Elaine Epperson, B.S. (Ph.D. student)

Report of Inventions. Not applicable.

Bibliography.

Gehnrich, S.C. and Aprille, J.R.: Hepatic gluconeogenesis and mitochondrial function during hibernation. Comp. Biochem. Physiol. B 91 (1988) 11-16.

Geiser, F.: Reduction of metabolism during hibernation and daily torpor in mammals and birds: temperature effect or physiological inhibition? J. Comp. Physiol. B 158 (1988) 25-37.

Heldmaier, G. and Ruf, T.: Body temperature and metabolic rate during natural hypothermia in endotherms. J. Comp. Physiol. B 162 (1992) 696-706.

Martin, S.L., Maniero, G.D. and Carey, C.: Patterns of gene expression in the liver during hibernation in ground squirrels. In: Geiser, F., Hurlbert, A.J. and Nicol, S.C. (Eds.), Adaptations to the Cold: Tenth International Hibernation Symposium. University of New England Press, Armidale, 1996, pp. 327-332.

Martin, S.L., Maniero, G.D., Carey, C. and Hand, S.C.: Reversible depression of oxygen consumption in isolated liver mitochondria during hibernation. Submitted to J. Comp. Physiol. B (1998) .